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Analysis of Expressions of the rII Gene Function of Bacteriophage T4

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Temperature-sensitive (*ts*) mutants of the T4 phage rII gene were isolated and used in temperature shift experiments that revealed two different expressions for the normal rII (rII$^+$) gene function in vivo: (i) an early expression (0 to 12 min postinfection at 30 C) that prevents restriction of T4 growth in *Escherichia coli* hosts lysogenic for λ phage, and (ii) a later expression (12 to 18 min postinfection at 30 C) that results in restriction of T4 growth when the phage DNA ligase (gene 30) is missing. The earlier expression appeared to coincide with the period of synthesis of the protein product of the T4 rIIA cistron, whereas the later expression occurred after rIIA protein synthesis had stopped. The synthesis of the protein product of the rIIB cistron continues for several minutes after rIIA protein synthesis ceases (O'Farrell and Gold, 1973). The two rII$^+$ gene expressions might require different molar ratios of the rIIA and rIIB proteins. It is possible that the separate expressions of rII$^+$ gene function are manifestations of different associations between the two rII proteins and other T4-induced proteins that are synthesized or activated at different times after phage infection.

The rII gene of bacteriophage T4 consists of two contiguous cistrons, rIIA and rIIB, the functions of which have not been explained. Mutations in either cistron can result in a number of effects on the physiology of phage growth in infected *Escherichia coli* hosts. Two types of observations suggest that the rII gene products play roles in T4 DNA metabolism. (i) T4 rII mutants do not propagate in *E. coli* hosts that are lysogenic for phage λ (2). It appears that normal T4 rII (rII$^+$) gene function is required to prevent the inhibition of T4 DNA replication that is caused, at least in part, by the *rex* gene function of λ prophage (1, 10, 11, 22, 26). (ii) T4 rII mutants can replicate their DNA and produce high yields of phage without the DNA ligase coded by T4 gene 30 (4, 6, 12). It appears that rII mutations result in decreased levels of DNase activity in infected cells and that this leads to decreased dependence on repair by ligase (13, 16, 23). T4 mutants that are defective in both gene 30 and the rII gene can utilize the host DNA ligase for growth (9).

Not all rII mutants show these two types of effects. For example, some rII mutations can rescue gene 30 defective phage without leading to restriction of phage growth in λ lysogens (4, 16). Also, some rII mutants are not restricted in λ lysogens although they exhibit the "rapid-lysis" phenotype that is characteristic of these mutants (5). In this communication we report on evidence that differences in phenotype among different rII mutants may be caused by alterations in temporal expression of the rII gene products in infected cells.

We isolated several temperature-sensitive (*ts*) rII mutants and used these in temperature-shift experiments that revealed two separable expressions for the rII$^+$ gene function in vivo. (i) An early expression (0 to 12 min postinfection at 30 C) that prevents restriction of T4 growth in λ lysogens. This expression seemed to coincide with the time of synthesis of the rIIA protein. (ii) A later expression (12 to 18 min postinfection at 30 C) that results in restriction of phage growth when the T4 gene 30 DNA ligase is missing. This expression seemed to require a higher rIIB to rIIA protein ratio than the earlier expression. The two separate expressions of rII$^+$ gene function could be shown to occur independently of each other and could be demonstrated by using *ts* mutations of either rII cistron. Different expressions of rII$^+$ gene function may be manifestations of different associations between the two rII proteins and between rII proteins and other T4-induced proteins that are synthesized or activated at different times after phage infection.

MATERIALS AND METHODS

Bacterial and phage strains. *E. coli* K38*str*^{r} and B^E are nonpermissive (*su*$^-$) for T4 amber (*am*) mu-

tants. *E. coli* K110*str*^{r} and CR63 are permissive (*su*$^+$3, tyrosine, and *su*$^+$1, serine, respectively). Some of the *E. coli* K-12 derivatives used had been cured of λ phage by heteroimmune superinfection with 21hyb$_2$ phage and relysogenized with a wild-type strain of λ(λ^{++}). The properties of these and other bacterial strains used have been described (14, 15).

Two double-*am* mutants of T4 gene 30 were used: *am*XE30 and *am*XC30. The genetic compositions and the growth properties of these mutants are summarized in Table 1. The T4 rII mutants that were used for genetic mapping experiments, were provided by S. Champe and have been described by Benzer (3).

Media and growth conditions. The liquid and solid media that were used in most experiments were as described previously (14, 15). The effects of Mg^{2+} on the rII mutant phenotypes were studied by using the growth conditions described by Garen (8) with the following modifications. Cells were grown to 10^8 per ml in 1% tryptone broth (Difco) containing 0.1 M NaCl and infected with phage at a multiplicity of infection (MOI) of 5 at 30 C. Anti-T4 serum (final k = 10) was added at 5 min postinfection to remove unadsorbed phage and after 2 min the infected culture was diluted 10^4-fold into 0.2% tryptone broth containing either 0.1 M $MgCl_2$ or 0.1 M NaCl. Phage growth was continued until the diluted cultures were lysed with chloroform at 2.5 h after infection. Infective center titers (at 7 min postinfection) and average phage yields per infected cell (burst size at 2.5 h postinfection) were determined on *E. coli* CR63.

The media and methods that were used for assays of T4 DNA and protein synthesis and for characterization of T4-induced proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis have been described (13, 14). [*Methyl*-^{3}H]thymidine and ^{14}C-labeled mixture of L-amino acids were purchased from New England Nuclear Corp., Boston, Mass.

Isolation of trsII mutants. We searched for T4 rII mutations that permitted the growth of gene 30-defective phage at 42 C, and restricted this phage at 30 C. Stocks of *am*XE30 and *am*XC30 were diluted and plated on *E. coli* K110 *str*^{r} and CR63, respectively. The plaques that formed after overnight incubation at 30 C were each picked and suspended in 1 ml of M9S growth medium containing a few drops of chloroform. These plaque solutions represented our random sample of double-*am* mutant phage. The average phage titer for the plaque solutions was about 5×10^7 PFU/ml. An appropriate amount of each of the plaque solutions was then plated on *E. coli* K38*str*^{r} and the plates were incubated at 42 C. Under these *su*$^-$ conditions *tsr*II mutations, as well as other types of rII mutations, permit *am*XE30 and *am*XC30 phage to form plaques. The plaques which did form after overnight incubation were picked and tested for growth on *E. coli* K38*str*^{r} at 30 and 42 C. Isolates that grew at 42 C but not at 30 C on K38*str*^{r} occurred at an average frequency of about 5%. The first such isolate to be encountered from each of the *am*XE30 or *am*XC30 plaque solutions examined was purified by replating on *E. coli* K38*str*^{r} at 42 C and a high titer phage stock was subsequently prepared from a sample of a single plaque that was grown at 30 C on *E. coli* K110*str*^{r} for *am*XE30 plaques and on CR63 for *am*XC30 plaques. Some of the *am*XE30/*tsr*II isolates that were obtained were backcrossed to wild-type phage and the single *tsr*II mutations were isolated. The single *tsr*II mutants were then crossed to *am*XC30 and the respective *am*XC30/*tsr*II mutants were isolated. These genetic purification procedures were carried out to remove secondary mutations that arose during the isolation of some of the *am*XE30/*tsr*II mutants that we studied.

Genetic mapping of tsrII mutants. Many of the *tsr*II mutants we isolated failed to grow on λ lysogens at 42 C and were mapped on EHA (Enriched Hershey agar) plates by using the deletion mapping methods described by Benzer (3). Some of our *tsr*II mutants, however, were not restricted in λ lysogens when grown on EHA agar plates at 42 C, but were restricted at this temperature on the lysogens in liquid media. So, it was possible to assign these mutants to the two rII cistrons by complementation tests that were carried out in liquid culture at 42 C. These tests consisted of coinfecting *E. coli* K38(λ^{++})*str*^{r} cells at an MOI of 5 with each of the *am*XE30/*tsr*II under examination and r638 (a deletion covering the entire rIIB cistron) or rH88 (a partial rIIA deletion mutation). Under these conditions, complementation between the *tsr*II under examination and the rII deletion resulted in rII$^+$ phenotype and in phage growth. Lack of complementation resulted in an rII$^-$ phenotype and in restriction of phage growth on the λ lysogen. All of *am*XE30/*tsr*II mutations we isolated complemented one or the other rII deletion used in the tests.

RESULTS

Genetic characterization of T4 tsrII mutants. All of the T4 *tsr*II mutants that are described here were isolated for their ability to grow in the absence of the T4 gene 30 DNA ligase at 42 C, but not at 30 C. These mutants were also examined for growth properties in λ

TABLE 1. *Growth characteristics of the T4 gene 30 amber mutants used*

Mutant	Growth[a] in response to					
	su$^-$		*su*$^{+1}$		*su*$^{+3}$	
	30 C	42 C	30 C	42 C	30 C	42 C
*am*XE30 (double mutant:*am*H39X /*am*E605)	−	−	−	−	+	−
*am*XC30 (double mutant:*am*H39X /*am*C104)	−	−	+	−	+	+
*am*H39X	−	−	+	+	+	+
*am*E605	−	−	+	−	+	+
*am*C104	−	−	+	+	+	+

[a] −, No growth; +, growth in spot tests on EHA plates.

lysogenic hosts. Table 2 summarizes the results that were obtained on a collection of *tsr*II mutants which were isolated in three different attempts at mutant isolation. The mutants could be classified into three groups on the basis of differences in their growth responses in λ lysogens. Group I mutants (Table 2) were restricted in λ lysogens at 42 C, but not at 30 C, group II mutants were not restricted in λ lysogens at either temperature, and group III mutants were restricted in λ lysogens at both temperatures. The existence of the *tsr*II mutants represented by groups II and III confirms that restriction in λ lysogens and rescue of gene 30-defective phage are two separable phenotypes of rII mutations (4, 16). It should be emphasized that the results shown in Table 2 describe the growth characteristics of the *tsr*II mutants on agar plates. In some cases, different growth responses were obtained by using liquid culture instead of agar plates. For example, in M9S and in EHA liquid media, all the group II mutants we isolated were restricted in λ lysogens at both 30 and 42 C. These effects appeared to be independent of the cistron in which the mutations occurred, and probably stem from some relationship between rII gene function and cell membrane permeability of T4-infected *E. coli* (7, 8, 18, 21, 25, 27).

Figure 1 shows the sites within the rII cistrons at which the *tsr*II mutations from experiment 1, Table 2 were found to map. The results from the somewhat limited number of mutations that have been examined so far suggest the existence of "hot spots" for temperature sensitivity within the rII gene, especially within the rIIA cistron. Few *tsr*IIB mutations have so far been isolated and mapped, but it may be significant to mention that all the group III mutations we have obtained mapped in rIIB. Group II muta-

TABLE 2. *Growth[a] characteristics of isolated tsrII mutants*

Group	No. of *tsr*II mutants[b] isolated per group			Growth of 30⁻/*tsr*II[c] phage on *E. coli*				Growth of 30⁺/*tsr*II[c] phage on *E. coli*			
				K-12		K-12 (λ)		K-12		K-12 (λ)	
	Exp 1	Exp 2	Exp 3	30	42	30	42	30	42	30	42
I	30	71	64	−	+	−	−	+	+	+	−
II	10	45	105	−	+	−	+	+	+	+	+
III	1	3	3	−	+	−	−	+	+	−	−

[a] −, No growth; +, growth in spot tests on EHA plates.
[b] The mutants for exp 1 were obtained from stocks of *am*XE30; those for exp 2 and exp 3 were from *am*XC30.
[c] 30⁻, *am*XC30 on *su*⁻ host; 30⁺, *am*⁺ for gene 30 or *am* gene 30 phage mutant on *su*⁺ host.

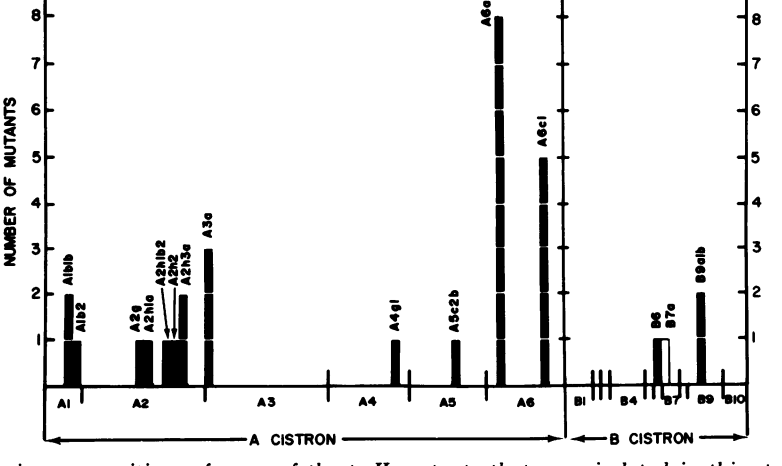

FIG. 1. *Genetic map positions of some of the tsrII mutants that were isolated in this study. The map positions of the 30 group I mutants (solid bars) and the group III mutant (open bar) from experiment 1 of Table 2 were determined by deletion mapping. The mutants at deletion segments A6a2a, A6c1, and B9a1b mapped at two sites within each of these segments.*

tions (not shown in Fig. 1) mapped in both rII cistrons (see Materials and Methods).

Other evidence for separable expressions of T4 rII$^+$ gene function. It has been demonstrated that the restriction of T4 rII mutants in λ-lysogenic hosts can be reversed in the presence of 0.1 M Mg^{2+} (8). The results we show in Table 3 confirm these observations, but also show that Mg^{2+} does not reverse the effects of rII mutations on the growth of gene 30-defective T4 phage. In the λ lysogen, *E. coli* K38(λ^{++})*str*^{r}, the T4 rII deletion mutant r1605 failed to grow in 0.1 M NaCl medium but exhibited a considerable degree of growth in medium containing 0.1 M $MgCl_2$ in place of the NaCl. This phenotypic reversal of rII$^-$ to rII$^+$ did not result in restriction of ligase-defective phage [*am*XC30/r1605 on *E. coli* K38(λ^{++})*str*^{r} and K38*str*^{r} hosts, Table 3]. These results suggest that the two phenotypes of rII mutations which we examined are due to different postinfection metabolic events.

Time of expression of T4 rII gene function. We used the *ts*rII and *am*XC30/*ts*rII derivatives that we isolated (Table 2 and Fig. 1) in a large number of temperature-shift experiments that were intended to measure the time of expression of the rII gene in infected cells. Mutants of group I could be used to measure the effects of shutting off rII$^+$ gene function on both, restriction in λ lysogens, and survival of *am*XC30 under *su*$^-$ conditions. Mutants of group II and III were only informative with regards to the effects on *am*XC30. Qualitatively similar results were obtained among mutants of any one of the three groups. The quantitative differences we did observe within groups were due to the different degrees of leakiness exhibited by the different mutants. That is, some mutations were more temperature-sensitive than others. We emphasize here the qualitative aspects of T4 rII$^+$ gene expression.

The experiment described in Fig. 2 demonstrates typical responses that were obtained with group I mutants. In this experiment one of the *am*XC30/*ts*rII isolates from group I and the corresponding single *ts*rII mutant were used to infect *E. coli* K38*str*^{r} and *E. coli* K38(λ^{++})*str*^{r} cultures at 30 C, and at various times after infection the cultures were diluted in growth medium at 42 C (to inactivate the rII$^+$ function). The rates of thymidine incorporation after the temperature-shift were then measured. The results (Fig. 2) demonstrated the following properties for the rII$^+$ gene function under the experimental conditions used: (i) rII$^+$ gene function is not required for growth in the λ lysogen after 12 min postinfection [*ts*rII-infected K38(λ^{++})*str*^{r} curve, Fig. 2], (ii) rII$^+$ expression before the 12th min postinfection does not lead to restriction of *am*XC30 (curve for *am*XC30/*ts*rII-infected K38*str*^{r}, Fig. 2), and

TABLE 3. *Effect of magnesium on the T4 rII mutant phenotypes*

Phage strain	*E. coli* host	Burst size in medium containing	
		0.1 M Na^+	0.1 M Mg^{2+}
T4D^{2+}	K38*str*^{r}	42	31
*am*XC30	K38*str*^{r}	<0.5	0.36
*am*XC30/r1605[a]	K38*str*^{r}	28	46
r1605	K38*str*^{r}	36	35
T4D^{2+}	K38(λ^{++})*str*^{r}	15	31
*am*XC30	K38(λ^{++})*str*^{r}	0.36	0.36
*am*XC30/r1605	K38(λ^{++})*str*^{r}	<0.36	10
r1605	K38(λ^{++})*str*^{r}	<0.36	30

[a] r1605 is an rII gene deletion that covers portions of both rII cistrons (3).

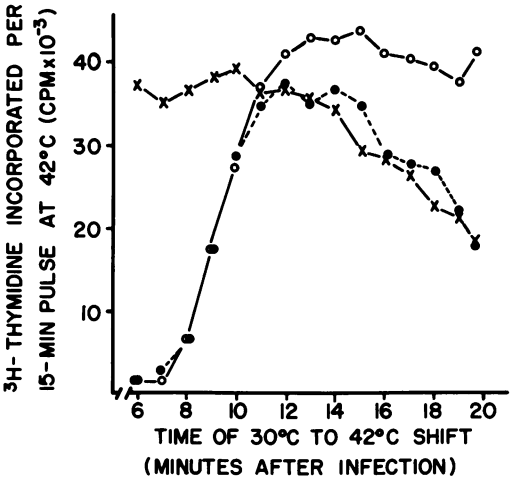

FIG. 2. *Time of expression of the T4 rII$^+$ gene. Log-phase E. coli cells (either K38str^r or K38(λ^{++})str^r) at 10^8 per ml were infected with the phage under examination at MOI 10 in M9S at 30 C. At various times postinfection, samples of infected culture were diluted 10-fold in M9S at 42 C. Fifteen minutes after the temperature shift, 1.6 μCi of [methyl-^{3}H]thymidine (at 2.5 μCi of ^{3}H/μg of thymidine) were added per ml of heated culture and thymidine incorporation was permitted to occur for an additional 15 min before it was stopped with 5 ml of cold 10% trichloroacetic acid per ml of incorporating culture. Symbols: (●) amXC30/tsrII-infected K38(λ^{++})str^r; (○) tsrII-infected K38(λ^{++})str^r; (X) amXC30/tsrII-infected K38str^r. The tsrII mutation used for this experiment mapped in T4 rIIA segment A6a2a.*

(iii) in rII$^+$-infected cells, the T4 gene 30 ligase is required for phage growth after the 12th min postinfection [compare *tsr*II-infected K38(λ^{++})-*str*^{r} and *am*XC30/*tsr*II-infected K38(λ^{++})*str*^{r}]. Similar results were obtained by using M9 medium that was not supplemented with amino acids and *E. coli* B, instead of *E. coli* K-12 hosts (data not shown). For example, at 30 C and in M9 (instead of M9S) medium, an *am*XC30/*tsr*II-infected *E. coli* B(λ) culture gave lower levels of thymidine incorporation than was obtained in the experiment for Fig. 2, but the timing of rII$^+$ expression was the same for the two experiments.

In other experiments, such as the one described in Fig. 3, we determined that thymidine incorporation was linear during the period of pulse labeling that was used for the experiment in Fig. 2. Cumulative DNA synthesis was measured in *am*XC30/*tsr*II-infected K38(λ^{++})*str*^{r} cells. When these infected cells were shifted to 42 C, there was an immediate effect on the amount of DNA which was subsequently synthesized. The accumulation of label into DNA was linear for at least 50 min. The rate of DNA synthesis (slopes of curves in Fig. 3) depended on the time of temperature-shift suggesting that there is a direct relationship between the extent of rII$^+$ expression and (i) the degree of restriction on a λ lysogen and (ii) the degree of restriction of ligase-defective phage.

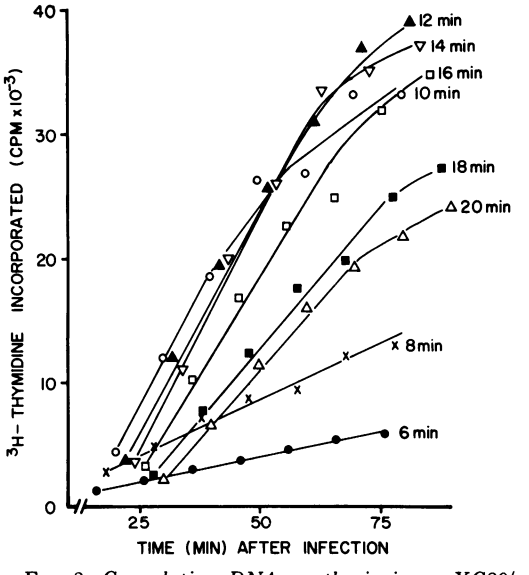

FIG. 3. *Cumulative DNA synthesis in amXC30/tsrII-infected E. coli K38(λ^{++})str^r. Log phase cells (at 10^8 per ml) were infected at MOI of 10 in M9S at 30 C. At various times postinfection samples of infected culture were diluted 10-fold into [methyl-^{3}H]thymidine (20 μCi of ^{3}H/ml; 27 μCi of ^{3}H/μg of thymidine) in M9S at 42 C. The times of temperature shifts are indicated on the thymidine incorporation curves. Samples (0.05 ml) were withdrawn from each diluted culture at 10-min intervals after dilution into isotope at 42 C and were assayed for trichloroacetic acid-insoluble radioactivity. The tsrII mutation used for this experiment mapped in T4 rIIA segment A6a2a.*

Time of synthesis of rII proteins. It has been shown that in T4-infected *E. coli*, the rIIB protein is translated from two mRNA species that are the result of two different modes of transcription (20, 24). We compared the kinetics of synthesis of the rIIA and rIIB proteins in an attempt to correlate the two expressions of rII$^+$ with the relative dosage of the two rII proteins. The levels of syntheses of the rIIA and rIIB proteins in *am*XC30-infected *E. coli* B^E cells were measured by using gel electrophoretic assays, the results of which are shown in Fig. 4. These results (Fig. 4) confirm the observation made by O'Farrell and Gold (20) that, in T4-infected cells, the rIIB protein continues to be synthesized for several minutes after rIIA protein synthesis has stopped. Under the growth conditions used in our study, the period of synthesis of the rIIA protein (Fig. 4) seemed to coincide with the period of rII$^+$ gene expression that is required to prevent restriction of T4 growth in λ lysogens (Fig. 2). So it appears that the two expressions of the rII$^+$ gene function occur at different rIIB to rIIA protein ratios.

DISCUSSION

Many different effects of T4 rII mutations have been described (2, 4, 12, 17, 18, 23, 25), but it has not been possible to relate all these effects to one metabolic deficiency. The restriction of T4 rII mutants in λ-lysogenic *E. coli* hosts appears to be due to metabolic events that occur during the first few minutes after phage infection. This has been determined in two types of study. (i) Certain concentrations of Mg^{2+} (8) reverse the restriction of rII mutants in λ lysogens only when added before 10 min postinfection at 37 C. (ii) Inactivation of *ts* T4 rII gene function prior to 10 to 20 min postinfection (at 30 C) results in restriction of phage growth in λ lysogens (19). Our studies confirm these observations and extend them by showing that a different T4 rII$^+$ gene expression is involved in the restriction of DNA ligase (gene 30)-defective phage.

In the study that was described in this report we examined the relationship between two rII mutant phenotypes to each other and to the patterns of synthesis of the phage rIIA and rIIB proteins in vivo. Our experiments defined two

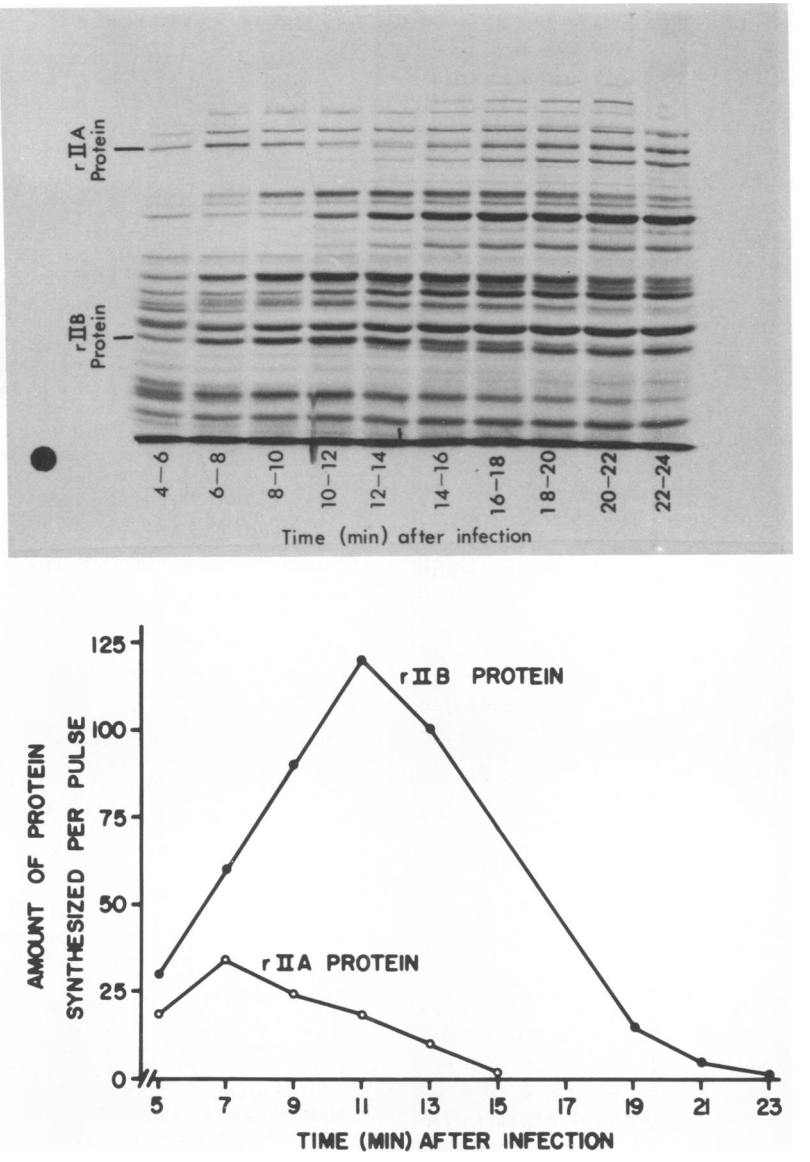

FIG. 4. *Patterns of rIIA and rIIB protein synthesis in T4 amXC30-infected E. coli B^{E}. Cells grown in M9 medium to a density of 2 × 10^{8} ml were infected at MOI of 10 with amXC30 at 30 C. At various time intervals postinfection, 1-ml samples of infected culture were pulse-labeled with 1 μCi of ^{14}C-labeled amino acids. Extracts of the isotopically labeled cells were then prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoretic assays by using the methods previously described (13). The figure shows a picture of an autoradiogram of a 7.5% gel and the results of densitometric tracings that were carried out on the rIIA and rIIB bands in the autoradiogram. The "amount of protein synthesized per pulse" refers to the weight (milligrams) of the rIIA and rIIB bands that were recorded on chart paper in the densitometric scanning of the autoradiogram.*

separate expressions of the T4 rII^{+} gene function that are not dependent on each other. In M9S medium at 30 C, the rII^{+} gene function that is required to prevent restriction of T4 in *E. coli* strains lysogenic for λ phage was expressed before the 12th min postinfection. Under the same conditions, the expression of rII^{+} gene function that results in restriction of DNA ligase-deficient phage occurred predominantly after the 12th min postinfection.

The pleiotropic effects of T4 rII mutations may be manifestations of the participation of

rIIA and rIIB proteins in several different phage-induced metabolic processes. It is likely that both the rIIA and the rIIB proteins participate in the same metabolic processes since all the rII mutant phenotypes that have so far been discovered can be exhibited by mutations in either rII cistron. The relative contributions of the two proteins in these processes, however, may differ and may be controlled by the different modes of synthesis of these proteins in vivo. The two effects of rII^+ gene function that we examined probably arise from two separate metabolic events since (i) not all rII mutations affect both rII^+ gene expressions (4, 16; Table 2) and (ii) a change in physiological conditions, i.e., the addition of 0.1 M Mg^{2+} to T4 rII-infected cells, affected one rII mutant phenotype (restriction in λ lysogens) but not the other (growth of gene 30-defective phage) (Table 3). The molar ratio of the rIIA and rIIB proteins changes progressively during early times after phage infection (20; Fig. 4). It is interesting to note that the time of synthesis of the rIIA proteins seems to parallel the rII^+ expression that prevents restriction of T4 growth in λ lysogens, whereas the restriction of DNA ligase-defective phage occurs at a later time, during a period of continued rIIB protein synthesis. Conceivably, the two expressions of rII^+ gene function that we examined require different rIIB to rIIA molar ratios.

It is possible to explain the two separate expressions of rII^+ gene function on the basis of both (i) the interaction of rIIA proteins with rIIB proteins, and (ii) the interaction of rII proteins with other T4-induced proteins. It has been shown that both the rIIA and rIIB proteins are membrane bound (7, 21, 27), but no free rIIA-rIIB protein complexes have yet been isolated. Since many of the reactions that are involved in macromolecular biosynthesis in T4-infected cells are thought to be membrane associated, it can be postulated that the rII proteins exert their effects on these processes either directly by acting as an enzyme (e.g., a controlled endonuclease [4, 12, 23] that acts on membrane-bound DNA) or indirectly by influencing the activities of various components in membrane-protein complexes.

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